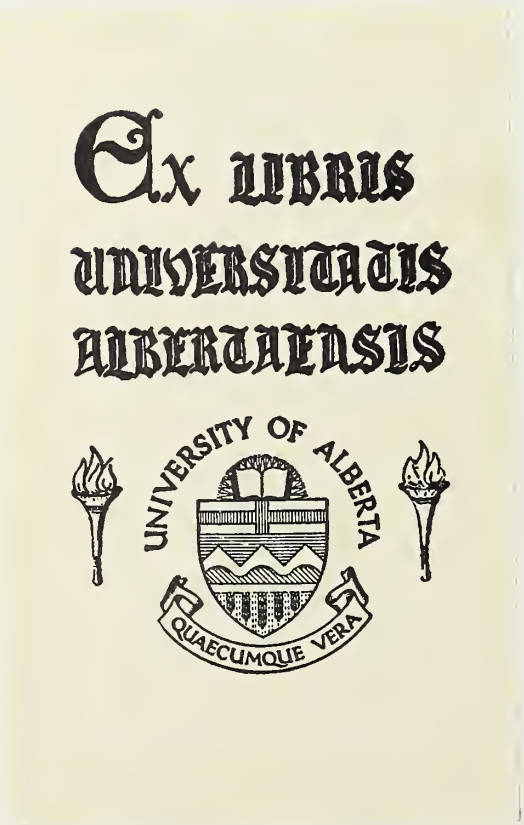

THE UNIVERSITY OF ALBERTA

THIRD DIVISION SEGREGATION FOR BISEXUALITY AT THE MATING-TYPE LOCUS OF *NEUROSPORA CRASSA* AND ITS GENETIC IMPLICATIONS

by

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ABSTRACT

The results of matings between a bisexual strain of Neurospora crassa and standard mating-type tester-strains are presented. Tetrad analysis of these crosses indicated that occasional third-division types of asci occur, showing segregation for the mating-types A/a, A and a in a ratio of 1:3:4. Furthermore they cannot originate by recombinational mechanisms such as "copying choice" and its modifications, nor by postulating a disomic or a gross chromosomal rearrangement involving the mating-type locus. The only recombinational model which fits the experimental data, is the "polaron hybrid DNA" theory as advanced by Whitehouse.

In addition, the bisexual strain exhibits somatic segregation in conidia for four mating-type reactions: A/a, A, a and "neutral." An extension of the "polaron hybrid DNA" theory has been proposed to facilitate an explanation of the mechanism underlying somatic recombination in the vegetative part of the fungus.

A compound structure of the mating-type locus is anticipated on the basis of the experimental results.

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INTRODUCTION

The phenomenon of recombination in the Ascomycete *Neurospora crassa* is usually accounted for on the basis of different modes of chromosomal exchange. Although Belling (3) related the phenomenon of recombination to the process of chromosome duplication, it was Darlington (6) who drew attention to the chromosome mechanics during meiosis and used this information for the formulation of his *theory of genetic exchange* which makes use of the *breakage and reunion* mechanism. The basis for this theory, however, can be traced back to Janssens (14) and Sax (39) and Morgan and his associates (48, 63), and is known as the *chiasma-type theory*.

According to Darlington (6, 7) chiasmata are (i) the consequence of genetic crossing over and (ii) a prerequisite for normal segregation of homologous chromosomes at meiosis, or, in other words: "crossing over is a condition of haploidisation" (52, p. 364). Stern (47) using *Drosophila* and Creighton and McClintock (5) using *Zea*, found that genetic crossovers were precisely correlated with cytological crossovers, and their work formed the basis for the theory of *non-allelic* or *classical crossing over*, which assumes that crossing over occurs between mutants belonging to two different cistrons.

Neurospora genetics up to 1955 dealt exclusively with problems associated with *non-allelic* or *classical* crossing over. In 1955, however, Mary Mitchell (23, 24) published data on inter-crosses of *Neurospora pdx* mutants, which showed that *pdx*$^+$ recombinants

do not always arise by reciprocal (i.e., classical) crossing over and it was thought that gene conversion (57, 20) underlay the formation of these segregationally aberrant asci. Mitchell (loc. cit.), in her experiments, used two pyridoxine-less mutants belonging to the same cistron, and hence in these crosses genetic exchange took place within the same functional genetic unit. Westergaard (52) refers to such an exchange as an allelic crossing over event, thereby recognizing that allelic crossing over is predominantly non-reciprocal.

The finding of non-Mendelian segregation ratios in asci of N. crassa has given rise to many theories with regard to the possible origin of these aberrant segregational products. All these theories are related to the one proposed by Belling (3), which explains recombination on the basis of chromosome duplication. A modern modification is known as the copy choice mechanism (15). According to theory a daughter chromosome is formed by the alternate use of the "paternal" and the "maternal" chromosome. Hence, when completed, the daughter chromosome is exactly like the "paternal" chromosome except for a segment that was replicated using the "maternal" chromosome as a template, and vice versa. It is evident, therefore, that the main difference between non-allelic crossing over and copying choice is that: "following breakage and reunion, the recombinant chromosomes inherit physical material from the two parental chromosomes; on the contrary copying choice yields recombinant chromosomes which are synthesized from new material and inherit only genetic information from the parental structures" (13, p. 339).

In order to accommodate other phenomena associated with allelic crossing over such as negative interference, and polarised crossing over (33, 31), the copying choice model has undergone many revisions (11, 34, 35, 42, 43, 45, 55).

Although there is no doubt that both allelic as well as non-allelic crossing over are operative in Neurospora, we do not know whether a single or a multiple mechanism gives rise to them. In case a common mechanism underlies both to be possible, it is necessary for the stage of homologous pairing to coincide with the time of chromosome replication. Pontecorvo (32) as well as Pritchard (35) are the main supporters of the above model and reject the classical chiasma-type theory. They propose that replication crossing over (occurring during pre-meiotic interphase and assuming a diploid interphase stage in the life cycle of Aspergillus) gives rise to allelic as well as non-allelic crossovers. Westergaard (52) in his review states that the chiasma-type theory is too well documented for repudiation. The classical experiments with Callimantis (40, 53), in which a correlation was shown to exist between sex-restricted crossing over and achimasmatic meiosis, provide strong evidence for the chiasma-type theory of crossing over.

With regard to Neurospora the controversy above outlined takes the form of two main alternatives (52):

1. There are no diploid divisions in the ascogenous hyphae.

In this case chromosome replication could have taken place either during meiosis, or before karyogamy. Pontecorvo (32) and

Pritchard (34, 35) suggest that chromosome replication may take place during meiosis (pachytene), and the occurrence of replication crossing over would enable allelic as well as non-allelic crossing over to take place simultaneously[1].

With replication before karyogamy, replication crossing over would be impossible, since members of homologous pairs of chromosomes remain isolated in different nuclei.

2. Diploid mitotic divisions occur in the ascogenous hyphae.

Mitchell (25, 26) indicated the possibility of pairing of homologous chromosomes in normally haplontic species (N. crassa). Accepting moreover, that classical non-allelic crossing over is due to chiasmata, it follows that non-allelic crossovers cannot be due to a chiasmata in a chiasma-type theory. Since it has been postulated that diploid mitotic divisions might occur it has been suggested (52) that a mechanism similar to that of replication crossing over may bring about allelic crossovers. This latter mechanism (duplication crossing over) is supposed to take place in the pre-meiotic (i.e., mitotic) cell, and is directly related to the process of chromosome duplication prior to karyokinesis.

In this latter model allelic crossing over is explained by the mechanism of somatic crossing over. From an experimental

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[1] In order to distinguish clearly between pachytene replication and mitotic replication, the author defines pachytene replication as replication, and mitotic replication as duplication.

point of view it is difficult to design a genetic system which, on analysis, would enable a conclusion to be drawn with regard to the mechanism underlying aberrant or non-Mendelian segregation in the ascus of Neurospora. Both, aberrant recombination and mitotic (somatic) recombination in the vegetative structure of the mold, are extremely rare phenomena. Nevertheless, there is a mutation which exhibits these characteristics quite frequently. The bisexual mutant FGSC 991, which is sexually compatible with either mating type of N. crassa (A and a), will recombine somatically into A or a with ease. As far as can be determined from literature, this mutant (developed by the Department of Genetics, University of Alberta) is unique for its mutation at the mating type locus as well as for its genetic behavior. The availability of genetic markers, both close and distant to the mating type locus, makes this mutant extremely useful for genetic analysis.

Bisexuality in itself has often been thought to be a genetic expression, which on analysis could contribute to a better understanding of the mechanics and physiology of heterokaryotic sexuality in fungi. As will be shown later, bisexuality in N. crassa can be due to different genetic causes. Therefore, the underlying thesis will deal with the mechanics of the inheritance of this mutation followed by a discussion of its mode of phenotypic expression.

LITERATURE REVIEW

The discovery of sexual interaction in fungi was made by Blakeslee in 1904 (4) when he established obligatory cross-mating in the "black bread mold," Rhizopus nigricans. In Blakeslee's terminology interaction between two self-sterile strains was called heterothallism, whereas sexual interaction between elements of a single, self-fertile strain, showing no differentiation in mating capacity among individual nuclei, was called homothallism (primary homothallism). Although Blakeslee (4) emphasized the occurrence of sexual dimorphism among obligatory cross-mating fungi it has since become clear, that sexual dimorphism is a rare characteristic among heterothallic fungi.

The classification of homothallism vs. heterothallism is perhaps not as absolute as it would appear to be from the original definitions. From a genetic point of view there are at least two conditions contributing to the loss of distinction between heterothallism and homothallism, which are of interest. In the four-spored species Neurospora tetrasperma (8) and Gelasinospora tetrasperma (10), for instance, there exists differentiation for sexual competence among individual nuclei: both species are self-sterile but sexually compatible. By means of incorporation of two meiotic products within the same spore, a stable sex-heterokaryon results with homokaryotic characteristics. The eight-spored variant of N. tetrasperma, on the other hand, is a typical heterokaryotic organism. Dodge (9) refers to the four-spored variant of N. tetrasperma as being

pseudohomothallic whereas Whitehouse (54) calls it a secondary homothallic variant.

Another condition which has genetic implications, is the mutation of a self-fertile (homothallic) species to a self-sterile but cross-fertile species. Related to this change is the mutation in a heterothallic species (N. crassa) which renders it now compatible to either mating-type (A and a), but preserves its self-sterile characteristic. This latter mutant can be described as a bisexual, but self-sterile, variant.

As Raper (37) points out, the control of sexual processes is imposed at two different levels:
"(1) the basic genetic predestination of future sexual capacities and (2) the genetic metabolic apparatus required for normal sexual development. The pattern of sexuality is imposed as a primary genetic control and is fixed at meiosis by the segregation of (a) neither sexual nor incompatibility factors, (b) sexual factors, or (c) incompatibility factors. The first of these determines universal compatibility, homothallism; the latter two determine two types of heterothallism" (51, p. 165-166) (viz., sexual morphological heterothallism and physiological heterothallism).

According to Raper and Esser (37), N. crassa belongs to the group of heterothallic fungi, in which the restriction of mating competence is due to incompatibility factors (physiological heterothallism), giving rise to a typical rigidly self-sterile and cross-fertile species. Yet, as Moreau and Moruzi (27) pointed out,

each physiological "sex" of N. crassa is morphologically a hermaphrodite. This conflict between physiological "sex" and morphological "sex" has lingered through Neurospora literature for decades, and although not resolved, the issue is currently avoided by adopting the term "mating-types" to describe the genetic factors A and a and relinquishing the use of the term "sex" for them. Summarizing, then, mating-types are physiologically diverse types which must be brought together to obtain mating. The term carries no implications as to sex.

The view expressed by Raper and Esser (37) that physiological heterothallism (as in N. crassa) is due to incompatibility factors is identical to the one expressed by Sansome (38). However, it seems that these physiological factors are not the only kind of compatibility factors involved. As Lindegren (18) pointed out, in bisexual strains of N. crassa, heterokaryons for A and a may be sexually self-incompatible but cross-fertile. The sexual control of such a system would require additional compatibility (or incompatibility) factors. Furthermore, the recent discovery that heterokaryosis itself is gene-controlled (so-called protoplasmic compatibility, brought about by het alleles (12)), makes the above system even more complicated.

Olive (30), in his essay on the evolution of heterothallism in the fungi, concluded that the most likely explanation of the origin of one-locus, two-allelic heterothallism is still to be found in the compound locus concept. However, a direct implication of the acceptance of this view would be that recombination of the pseudoalleles could give rise to a small number of homothallic

descendants. Olive (30) states that St. Lawrence (see 30) obtained several single-ascospore isolates of N. crassa which, although not self-fertile, could cross with either the A or the a strain. The suggestion was made that these ascospores contain disomic nuclei carrying a homologous pair of chromosomes. This assumption, however, carries the inevitable consequence that such isolates would be self-fertile, unless a second requirement, the ability of self-fertilization, has not been met. Therefore, the observations by St. Lawrence (see 30) indicate a much more elaborate system of sexual control than the one outlined by Olive (30). Lindegren (18) reported on an unusual ascus of N. crassa containing four bisexual and four akaryotic spores. The mycelia from each of the four bisexual spores produced conidia asexually (as well as perithecia sexually). Cultures were made from single conidia of each of the four lines. Since conidia are usually multinucleate, most of the mycelia grown from the four lines would be expected to be bisexual. This was the case, but unisexual conidia were also produced in each instance. Asci produced directly by the four bisexual mycelia were analyzed and no bisexuals were encountered. Several matings were also made between the unisexual strains obtained by somatic segregation from the sex-heterokaryons. No bisexual ascospores were found among their offspring. According to Lindegren (18) these experiments suggest that the third division in the ascus may, in some cases, function as a device to insure incorporation of two nuclei of opposite sex in each ascospore. A similar case was observed by Moreau and Moruzi (27) in N. sitophila. Four-spored asci were found with large ascospores, homothallic in their mating type reaction.

Recently, Newmeyer (29) reported on bisexuality in N. crassa resulting from crossing an arg-1 strain (H 4250) to the wild type. About 1/4 of the viable ascospores initially yielded characteristic inhibited colonies which formed dark pigment on complete medium (so-called "Dark Agars" or DA). After several days the DA's "escaped" from the inhibition and assumed a growth like the wild type. Most DA's yielded abundant perithecia when crossed to either A or a mating type, whereas conidial plating showed that escaped DA's contained a mixture of A and a conidia. Since abortion patterns were observed in the asci (6 viable, 2 non-viable) a pericentric inversion is indicated. Newmeyer (29) suggested that this chromosomal aberration could yield a viable duplication-deficiency class among the segregants, and a non-viable reciprocal duplication deficiency is non-viable. The initial DA phenotype is attributed to heterozygosity of the mating type locus and the "escape" from inhibition is attributed to formation of homozygous A and a nuclei, probably by mitotic crossing over.

MATERIALS AND METHODS

1. Materials

The strains of Neurospora crassa used in the following experiments were: the UV-induced bisexual strain FGSC 991 (originally developed by Mr. Paul Unrau, from strain FGSC 262 A), the standard St. Lawrence strains FGSC 262 A and FGSC 533 a, and the tester strains FGSC 257 and FGSC 247 (see Table I).

2. Media

The following media were used:

Standard complete medium

All cultures were maintained on the standard complete Neurospora medium as described in the "Stanford Neurospora Methods" (46).

Modified Westergaard's synthetic crossing medium

Ryan's modification of Westergaard's medium as described in the "Stanford Neurospora Methods" (46) was used as a crossing medium for sexual reproduction and for mating type tests.

Sorbose plating medium

This medium causes Neurospora to grow in a very restricted colonial form allowing single colony isolation. The medium contains liquid Vogel minimal medium (50) supplemented with 2% agar, 1% sorbose and 0.1% sucrose. The sorbose and sucrose were sterilized separately to prevent caramelization.

Table 1. List of strains employed and their characteristics

Stock number	Markers	Linkage group	Centromere distance		Remarks
			Value	Range*	
FGSC 262	Mating type, A	IL	6.6	6.1 - 7.2	Vegetative re-isolate of "St. Lawrence's" 74A. Good conidiation and aerial growth (Barratt and Ogata, 1962).
FGSC 533	Mating type, a	IL	as above		Vegetative re-isolate of "St. Lawrence's" 79a. Good conidiation and aerial growth (Barratt and Ogata, 1962).
FGSC 257	Leucine, crisp and mating type: leu-3, cr, A	IL, C	leu-3 11.9	8.0 - 16.0	Leucine-less ("leaky"), early and uniform conidiation over agar surface (Barratt and Ogata, 1962).
			cr 4.6	3.5 - 5.7	
FGSC 247	Leucine, crisp and mating type: leu-3, cr, a	IL, C	as above		Leucine-less ("leaky"), early and uniform conidiation over agar surface (Barratt and Ogata, 1962).
FGSC 991	Bisexual, A/a		--		Poor conidiation, light orange pigment (Barratt and Ogata, 1962).

* Barratt et al., 1954.

Liquid Vogel medium

This medium is a "minimal" type medium containing inorganic salts, biotin, and sucrose, as described in the "Stanford Neurospora Methods" (46).

Agar substrate for manipulation and isolation of ascospores

Four percent agar suspended in water was autoclaved and poured into Petri dishes. Blocks of the solidified agar were removed with a scalpel for use in ascospore isolation.

Supplemental medium

L-leucine - 200 mg/1000 ml medium

Mating type test for individual conidia of the bisexual strain FGSC 991

Cultures of the bisexual strain FGSC 991 were grown on solidified minimal medium for about 6 - 7 days at 30° C. A conidial suspension was prepared by washing the conidia from the mature agar slants using cold sterile distilled water. After filtration through glass-wool filters, suitable dilutions of the conidial suspension (200 cells/0.1 ml) were made in cold sterile distilled water. Ten ml of each adjusted dilution were pipetted and mixed with 500 ml of sorbose plating medium at 45° C and distributed over 10 plates. After 3 days of incubation at 30° C, colonies were isolated and transferred to a complete medium. Conidial transfers from the above isolates were then tested for mating type.

Single random ascospore isolation

Crosses between strains were made in half-pint milk bottles containing liquid synthetic crossing medium. The method of ascospore isolation involves the use of blocks of the 4% solidified agar. The blocks were cut from solidified agar on Petri dishes and were placed under a dissecting microscope (10X). Before use, these blocks were superficially sterilized with 1.5% sodium hypochlorite solution (to kill adhering conidia, mycelial fragments and most other contaminants).

Single ascospores were then transferred with a glass-needle to the edge of the agar block, where they were lined up in a row, 2 mm apart and about 1 mm from the edge. With a glass transfer "lance" small blocks of agar (2 mm wide and 1/2 mm thick) were removed together with the adhering ascospore.

With a glass needle, these small blocks were transferred (spore side up), one by one, to small test tubes (7.5 x 100 mm) containing solidified complete medium. The isolates were heat-activated by placing the racks of culture tubes in a 60° C water bath for 30 minutes. Subsequently, the isolates were incubated at 30° C for about 406 hrs at 30° C in order to assure maximum ascospore germination. After this period of incubation, the cultures were stored at 4° C until needed.

Tetrad analysis

A method similar to that described under the section Single random ascospore isolation was used, except that complete eight-spored asci were dissected with a glass-needle by means of a distal rupture in the ascus sack. Complete asci were removed by squeezing the almost mature perithecium with forceps, thereby forcing the rosette of asci to separate from the main structure. Prior to heat-activation, the isolates were matured for about seven or more days.

Mating type tests

Pairs of large standard Petri dishes (150 x 25 mm), each containing about 50 ml synthetic crossing medium, were spread with a conidial suspension of the standard St. Lawrence tester strain (FGSC 262 A or FGSC 533 a). The inoculated plates were wrapped in paper towels and incubated for 7 days at 25° C to allow protoperithecia to form. After the initiation of protoperithecia, crosses were made by re-inoculation of the plates either with hyphal material or with a conidial suspension of each isolate to be tested. These plates were kept for approximately seven days at room temperature after which they were examined and rated.

Determination of linkage

In order to detect linkage and to estimate chromosome map distances, cultures from isolated ascospores from the cross FGSC 991 x FGSC 247 were tested on liquid Vogel medium. The presence

of a biochemical mutant was ascertained by the failure of the isolate to grow on liquid minimal medium. Morphological characters were studied after 24 hours and 48 hours of incubation at 30° C on standard liquid complete medium in 7.5 x 100 mm tubes, using cultures grown directly from ascospores. Centromere distances in Neurospora map units were calculated according to standard methods (23, 24).

Visual estimation of the number of protoperithecia in bisexual strains

Standard Petri dishes, each containing about 15 ml synthetic crossing-medium, were inoculated in the centre with the bisexual strain to be tested. Other Petri dishes (also containing 15 ml synthetic crossing medium) were inoculated either with the reference strain FGSC 262 or with FGSC 533 (St. Lawrence's wild type strains). Both sets of plates were incubated at 25° C for six days, after which the number of protoperithecia per given surface area, was counted under a dissecting microscope.

OBSERVATIONS

1. General remarks

Although the bisexual strain FGSC 991 will cross with either mating-type tester-strain FGSC 262 (A) or FGSC 533 (a), (both used as a protoperithecial strain) the amount of mature perithecia produced when crossed to an a strain exceeds by far, the number of perithecia resulting from the cross FGSC 991 x A. The reciprocal crosses, in which FGSC 991 is used as the protoperithecial strain and either FGSC 553 (a) or FGSC 262 (A) as the spermatial parent, are weak in their mating-type reaction, especially when the bisexual strain is fertilized by the a tester-strain. In the latter cross the reduction of mating-type reaction might be partly due to an impairment in the production of protoperithecia.

Table II lists protoperithecial counts per given surface area for all of the strains employed. As can be seen, the bisexual strain FGSC 991 produces only 3 perithecia per Petri dish surface, whereas the standard, wild-type strains (FGSC 262 and FGSC 533) produce 159 and 169 protoperithecia respectively. The bisexual re-isolates (15-1, 3-1) from the cross FGSC 991 x FGSC 533 produced 37 and 41 protoperithecia respectively. Both bisexual re-isolates cross well with tester-strain A as well as a.

All crosses involving the bisexual strain FGSC 991 and the tester-strains, produce a considerable number of non-viable ascospores (approximately 35%). No abortion patterns--typical for chromosomal aberrations--have been observed. Since completely

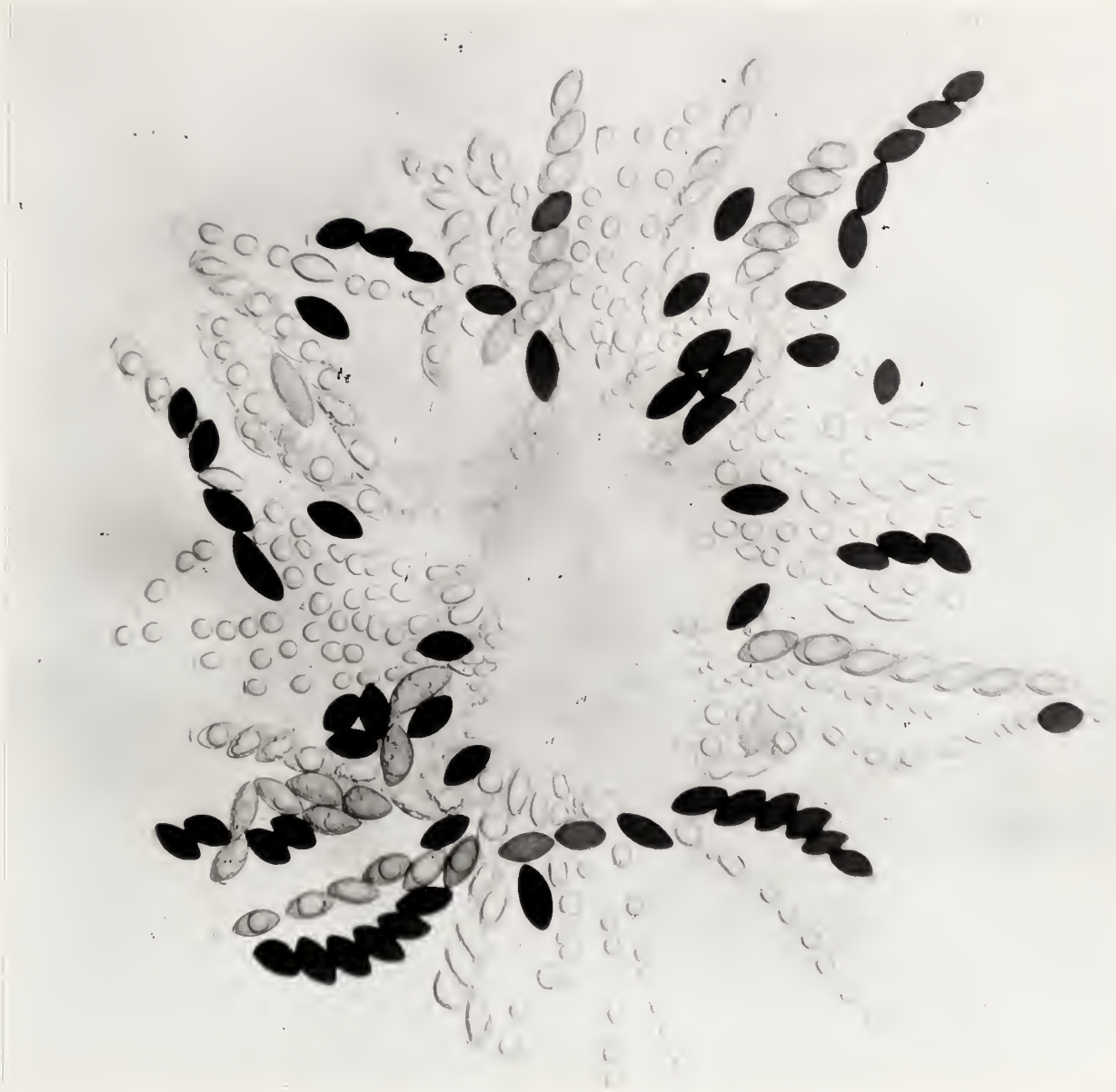
Table II. Protoperithecial counts, per Petri dish surface, for the strains employed

Strain	Phenotype	No. of protoperithecia per Petri dish surface
FGSC 262	Wild type, A	159
FGSC 533	Wild type, a	167
FGSC 257	Leucine-less, crisp, A	83
FGSC 247	Leucine-less, crisp, a	75
FGSC 991	Bisexual	3
15-1	Bisexual (re-isolate)	37
3-1	Bisexual (re-isolate)	41

viable, 8-spored asci have been found which exhibit segregation for bisexuality (although non-Mendelian), it is believed that no gross chromosomal rearrangement underlies the origin of bisexuality in strain FGSC 991. Figure 1 depicts a general view of a rosette of asci. (Colorless spores are non-viable.)

The bisexual strain FGSC 991 carries, in addition, a morphological mutation, which in many respects resembles peach (pe), a mutation for micro-conidiation. For instance, the phenotypic interaction of the unlocated morphological marker with fluffy (fl), an aconidial mutation, is similar to that betweel fl and pe. Although segregation for this marker has been followed in all the crosses, no use could be made of the data obtained, since the marker is not located in linkage group I.

Fig. 1. A rosette of asci from the cross FGSC 991 x FGSC 533. Note the large proportion of aborted spores. Magnification 400X.



2. Somatic segregation for mating-type among conidia of the bisexual strain FGSC 991

The somatic segregation frequencies, reported in Table III, were found among conidial isolates from strain FGSC 991 tested for mating-type (mt) by crosses to the wild-type tester-strains FGSC 262 and FGSC 533.

Table III. Mating-type test of somatic segregants among conidia of strain FGSC 991

Strain	No. of colonies isolated	Frequencies of genotypes (mt) A	a	A/a	Neutral[1)]
FGSC 991	220	8(3.6%)	110(50%)	6(2.7%)	96(43.7%)

1) Showing no mating type reaction with either A or a.

3. Random spore isolation from the cross FGSC 533 x FGSC 991

Crosses between the wild-type strain FGSC 533 and the bisexual strain FGSC 991 yielded large numbers of ascospores which germinated well (average germination frequency, 78%). Table IV lists the observed segregation data for mating-type. Since there appears to be a shortage of A segregants, the data are compared with crosses identical to the above, except that bisexual re-isolates of the cross FGSC 533 x FGSC 991, viz. isolates 15-1 and 3-1 were used instead of FGSC 991.

Two bisexual isolates appeared among the offspring of the cross FGSC 991 x FGSC 533, whereas only one appeared among each of the offspring of the crosses 15-1 x FGSC 262 and 3-1 x FGSC 533.

Table IV. Mating-type segregation data from random spore isolations from the cross FGSC 533 x FGSC 991

Cross	No. of single ascospores isolated	Germination of ascospores	No. of "segregants"		
			A	a	A/a
FGSC 533 x FGSC 991	500	71.4	165	190	2
FGSC 262 x 15-1	500	81.2	192	213	1
FGSC 533 x 3-1	500	81.4	205	201	1

4. Tetrad analysis of the cross FGSC 533 x FGSC 991

Out of a total of 30 asci dissected out, only 16 could be satisfactorily analyzed. Due to non-viability, 14 of the asci were unsuitable for classification. One bisexual segregant was found in an ascus showing the following genotypes in linear order:

1. A/a
2. A
3. A
4. A
5. a
6. a
7. a
8. a

Table V. Mating-type segregation data from tetrad analysis of asci from the cross FGSC 533 x FGSC 991

Cross	No. of asci isolated	No. of asci classified	No. of bisexuals	% of second division segregation asci for mt	C. o. units
FGSC 533 x FGSC 991	30	16	1	12.6	6.3

5. Random spore isolations from the cross FGSC 247 x FGSC 991

The percentage germination of ascospores originating from the cross FGSC 247 (*leu*$^-$-3, *A*, *cr*$^-$) x FGSC 991 (*leu*$^+$-3, *A*/*a*, *cr*$^+$) amounted to 66.7%. A complete analysis with respect to markers is given in Table VI. The calculated intercept distances are: 21.3 c.o. units for *leu*-3 and *mt* (expected (2): 5.3), 14.7 c.o. units for *cr* and *mt* (expected (2): 11.2) and 27.8 c.o. units for *leu*-3 and *cr* (expected (2): 16.5).

6. Recombination data for leu-3, mt and cr from tetrad analysis

Out of a total of 75 asci dissected, 47 were classified with respect to the segregation patterns of the markers concerned. The calculated centromere distances were found to be as follows (see Table VII):

mt 7.5 c.o. units (expected: 6.6)

leu-3 12.7 c.o. units (expected: 11.9)

cr 4.3 c.o. units (expected: 4.6)

One of the asci exhibited third division segregation for bisexuality resulting in a 1:3:4 ascus for A/a, A and a respectively.

Table VI. Recombination data for mt, leu-3, and cr from random ascospore isolations from the cross FGSC 247 x FGSC 991

Genotypes	No.	Recombination for		
		leu-3 and mt	cr and mt	leu-3 and cr
Parental				
leu^-, cr^-, a	147			
leu^+, cr^+, A	86			
leu^+, cr^+, A/a	2			
Single crossovers				
leu^-, cr^+, a	21		21	21
leu^+, cr^-, a	30	30		30
leu^-, cr^+, A	19	19		19
leu^+, cr^-, A	5		5	5
leu^+, cr^-, A/a	1		1	1
Double crossovers				
leu^+, cr^+, a	6	6	6	
leu^-, cr^-, A	16	16	16	
Total	333	71	49	76
Percentages		21.3	14.7	27.8
Expected		5.3	11.2	16.5

Table VII. Recombination data for leu-3, mt and cr from tetrad analysis of the cross FGSC 247 x FGSC 991

Cross	No. of asci isolated	No. of asci classified	No. of bisexual spores	Centromere distance (50% of second division asci, equivalent to c.o. units) for: mt	leu-3	cr
FGSC 247 x FGSC 991	75	47	1	7.4	12.7	4.3

DISCUSSION

1. The mechanism underlying bisexuality in Neurospora crassa in strain FGSC 991

Raper and Esser (37) in their review of the fungi state that incompatibility alleles are apparently very stable: "as no report of the mutation of the factors appears in the voluminous literature for Neurospora, Ascobolus, Venturia, etc.; neither is there published evidence of a compound incompatibility locus" (p. 169).

Although reports on bisexuality in N. crassa are rare, (see p. 5) there is no doubt that, occasionally, bisexuals have been observed. Yet, as Raper and Esser (loc. cit.) pointed out, they do not consist of mutations of the incompatibility alleles. The bisexual strains of Moreau and Moruzi (27), and of Lindegren (18), can be disqualified as mutants, since in both cases the change in genotype was not transmitted to the progeny. It is not known if the bisexual strain reported by St. Lawrence (see Olive, 30) yielded bisexual offspring. The recent report by Newmeyer (20) does not include a genetic analysis regarding mating-type of the offspring of the bisexual strain; therefore, we do not know if the bisexual characteristic is inherited or not. In the latter case, bisexuality was due to a pericentric inversion, and the viable meiotic cross-over product was deficient for the tip of the linkage group IR_s and duplicated (and usually heterozygous) for all of the IL group distal to the break-point. It is, however, doubtful that

of gross chromosomal aberration, as outlined above, would be inherited by a subsequent generation. This thesis, therefore, contains the first description to be found in the literature of a mutation of the mating-type locus (incompatibility alleles) and its subsequent inheritance by the progeny.

Bisexuality in N. crassa is of considerable interest to the geneticist, since the causes underlying the phenomenon can differ widely. In Lindegren's (18) example and also in the one reported by Moreau and Moruzi (27), bisexuality is due to the inclusion of two nuclei of opposite mating-type into one ascospore (mating-type heterokaryon). The bisexual strain reported by St. Lawrence, on the other hand, (see Olive, 30) is probably due to the formation of a disomic for the mating-type chromosome, whereas Newmeyer's bisexual strain is the result of a heterozygous duplication involving the mating-type locus.

Although strain FGSC 991 is a mating-type heterokaryon (as is evident from the conidial segregation data in Table III), its bisexual characteristic is not directly related to its heterokaryotic condition. The occurrence of occasional 8-spored asci (cross: FGSC 247 x FGSC 991 and FGSC 533 x FGSC 991) with a segregation pattern of 1 A/a : 3 A : 4 a, rules out the possibility that mating-type heterokaryosis underlies bisexuality in this strain.

As mentioned above, the somatic segregation for four mating-type reactions (i.e. A/a, A, a and "neutral") indicates that the vegetative structure of strain FGSC 991 is a mating-type

heterokaryon involving four different kinds of nuclei. Conidiation in bisexuals would thus give rise to "homozygous" microconidia (A/a, A and a) and a few "homozygous" macroconidia which, because of their multinucleate nature, can be expected to be rare. The remainder of the macroconidia are mating-type heterokaryons with different ratios of the constituant nuclei. This latter class is "neutral" in its mating-type reaction and differs in this respect from mating-type heterokaryons with a typical bisexual expression (18). Although bisexuality in strain FGSC 991 is not due to a mating-type heterokaryon, in the course of its vegetative growth it will give rise to one.

It is not believed that a chromosomal rearrangement underlies the bisexuality in strain FGSC 991. The shortage of A segregants (165 A : 190 a) from the cross FGSC 533 x FGSC 991 (Table IV) and also among the offspring of the cross FGSC 247 x FGSC 991 (126 A : 204 a, Table VI) would not be expected if a chromosomal rearrangement involving the mating-type locus were present. The tetrad analysis of the latter cross (Table VII) yields almost perfect values for the centromere distances of the markers involved, which cannot be expected in case a gross chromosomal rearrangement involves the mating-type locus. In addition, the bisexual re-isolates 15-1 and 3-1 show an expected mating-type ratio (192 A : 213 a and 205 A : 201 a respectively) among their offspring when crossed to the wild-type tester-strains.

The failure to detect abortion patterns in asci resulting from the crosses FGSC 533 x FGSC 991 and FGSC 247 x FGSC 991 is in

accordance with the above conclusion, namely that bisexuality in strain FGSC 991 (and also in strains 15-1 and 3-1) is not caused by a chromosomal aberration involving the mating-type locus. There does exist, however, a possibility that bisexuality in strain FGSC 991 is due to an incomplate disomic condition. In such a case, the disomic would be able to proceed through reduction division, yielding an A/a and an a nucleus after which the disomic would be only partly replicated (for instance, in the case of an acentric homologue carrying a). Division II would subsequently yield the following nuclei: A/a, A, a, a. Duplication prior to division III would again only duplicate A from the disomic A/a with the result that after division a pattern A/a, A, A, A, a, a, a, a is established. Hence, in this scheme only one complement (A) is replicated prior to mitotic division, and not the entire disomic (A/a). Nevertheless, one has to repudiate the evidence that a disomic is involved. On plating conidia 2.7% of the isolates are of an A/a genotype, indicating that the "apparent disomic" can be replicated somatically as A/a. Moreover, genotype A occurs in a frequency of 3.6%, a value which, on the assumption that A/a is replicated as A (i.e. the breakdown of the disomic into a monosomic), is much too low.

2. The mechanism underlying the origin of third division segregation for bisexuality in strain FGSC 991

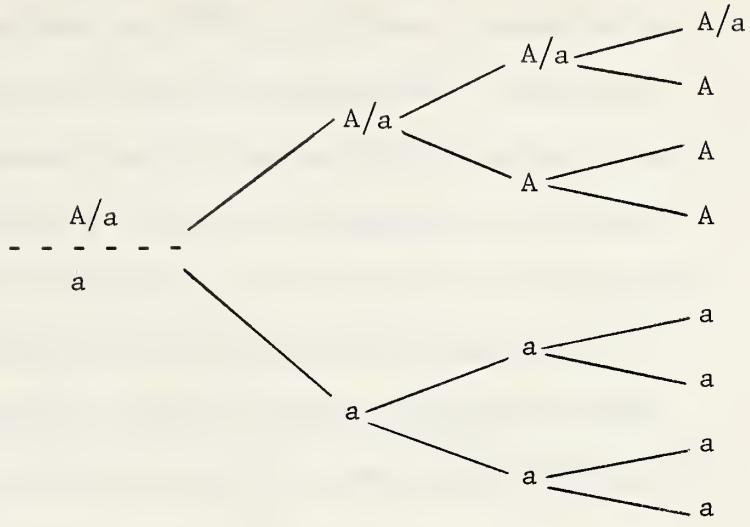
The somatic segregation data for conidia of strain FGSC 991 indicates that 4 phenotypically distinct genotypes originate: a, A, A/a and "neutral." Because karyogamy in Neurospora consists of fusion of two haploid nuclei (McClintock, 21), the descendents

of a particular ascus are determined by the protoperithecial strain (i.e. A or a) and only one of the above mentioned somatic nuclear products. Both tester-strains (FGSC 533 and FGSC 247), which have been extensively used in the genetic analysis of the bisexual strain FGSC 991, are of the mating-type a. Hence, the following mating combinations are possible:

Protoperithecial strain	Spermatial strain	Remarks
a	A	Expected to be fertile
a	A/a	Expected to be fertile
a	a	Expected to be sterile
a	"neutral" or "no sex"	Expected to be sterile

Because the above mentioned of the spermatial strains (conidial genotypes) A/a and A occur approximately in the same frequencies (Table III), the population of asci resulting from a cross between FGSC 991 and FGSC 533 (or FGSC 247) is expected to consist of approximately 50% of asci showing segregation for A and a and 50% of asci showing segregation for A/a and a. As can be seen from Tables V and VII, among a total of 63 (16 + 47) asci only 2 asci showed segregation for bisexuality (A/a). The reason for this low frequency of asci showing segregation for A/a will be discussed later (p. 35). Both asci showing segregation for A/a exhibited third-division segregation for this factor (i.e. non-identity of sister-spores), and are therefore conveniently called 1:3:4 asci. Although the fusion-nucleus resulted from karyogamy between an A/a and an a nucleus, it is clear that the A/a genotype

is not reproduced as such in the mitotic divisions following the reduction division. Instead, an A genotype is substituted:



The question can be asked, as to whether or not these occasional 1:3:4 asci can arise by any of the mechanisms so far proposed in the literature (p. 2). The "copying choice" model with a "double replication mechanism" does not explain without questionable modifications the origin of a 1:3:4 type of ascus. Its main value lies in the fact that an aberrant ascus showing a 2:6 pattern can be resolved by the model. In a 2:6 type of ascus, sister-spores are identical, and hence there is no evidence for third-division segregation. In order to meet the demand that the sister-spores in a 2:6 ascus are identical, DNA replication in the "copying choice" model takes place in a conservative manner. The model can, however, be modified in such a way that it facilitates an explanation for the origin of a third-division type of asci. The DNA replication in that case is required to take place in a semi-conservative manner (Taylor, 49; Pritchard, 34). In other words, two different segregation patterns each require a different

mode of DNA replication, or, as Pritchard states: "It is clear that at the present moment there is a contradiction between the genetic data from tetrads and existing evidence concerning the molecular structure and its mode of replication. Two obvious possibilities that might resolve this contradiction are: first, that replication of DNA at meiosis is conservative, not semi-conservative as it is at mitosis; or, secondly, that replication at meiosis is predominantly semi-conservative, but that a conservative distribution of DNA sub-units is imposed on DNA molecules which have been involved in recombination" (34; p. 173-174).

Due to the fact that the 1:3:4 type of ascus showing segregation for A/a, A and a is a recombinational ascus for A, showing in addition third-division segregation for A/a, the "copying choice" mechanism cannot underly its origin. A 1:3:4 type of ascus would require DNA replication to take place simultaneously in a conservative as well as in a semi-conservative manner.

The possibility suggested by Mitchell (25, 26) (based on a recombinational model by Weijer and Dowding (51), that somatic allelic exchange (and necessarily not reciprocal) underlies the origin of aberrant asci is also attractive, since in the case under discussion it may provide an explanation for the origin of the different conidial genotypes. This proposition, however, must be rejected, since in the origin of a 1:3:4 type of ascus segregating for bisexuality, the non-identity of sister-spores in these asci would require that somatic exchange extends into division III of ascogenesis. Unfortunately, the cytological evidence (Singleton, 41) does not substantiate such a postulate.

Whitehouse (55, 56) has advanced a crossing over model which assumes that non-allelic crossing over takes place at pachytene, according to the classical scheme of breakage and reunion. It is furthermore assumed that the process of chromatid reunion requires localized DNA synthesis at the point of breakage. Allelic crossing over (not necessarily reciprocal) would be possible at the point of breakage during repair of the broken chromatids. In the literature this model is known as the polaron hybrid DNA theory (Whitehouse, 55, 56). The steps in crossing over, according to this theory are shown in Figure 2. "In (i) are shown two homologous chromatids, each assumed to correspond to a DNA molecule. The horizontal lines represent nucleotide chains, the arrow-heads show the direction of the phosphate-sugar backbones and they also mark the position of the ends of a polaron[1]. The spiral coiling is not shown. The short vertical lines indicate hydrogen bonding between bases in complementary nucleotide chains. It is assumed that the initial step in crossing over is the breakage of the chains of opposite polarity, one in each molecule, at one end of the polaron and their separation from the complementary chains over the greater part of the length of the polaron (Fig. 2(II)). Synthesis of new chains, shown by broken lines in Fig. 2 (iii), is postulated to occur alongside the old unbroken chains and complementary to them, beginning at the end of the polaron and possibly extending unequally as in the diagram. The newly synthesized chains are then assumed to separate from their templates (Fig. 2(iv)) and each to pair with

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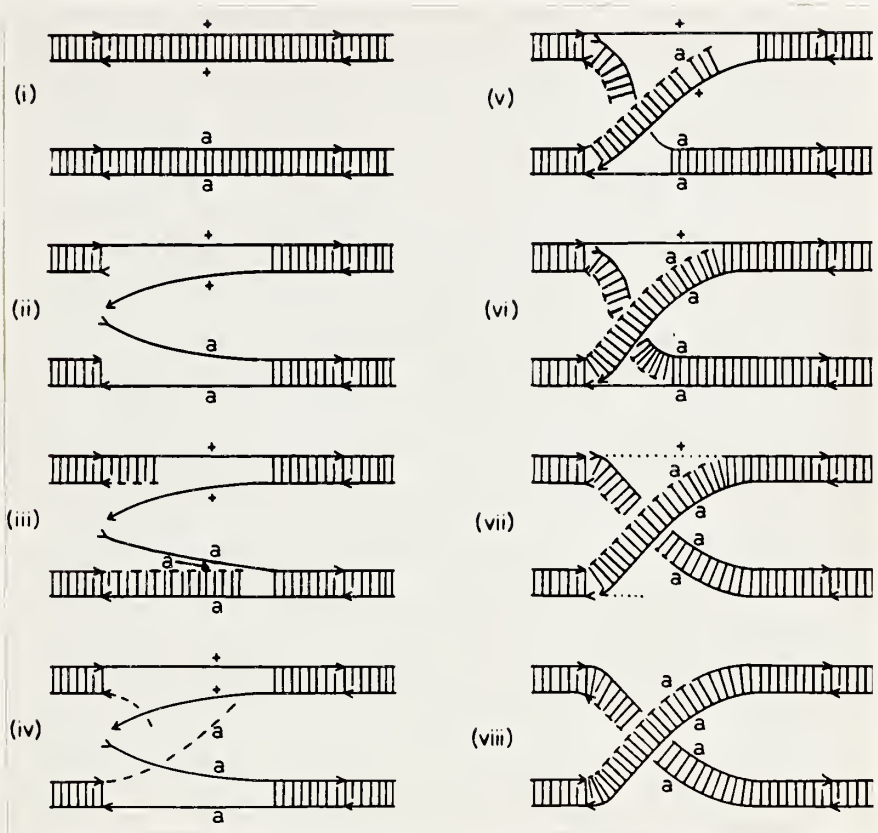
1) The term polaron was proposed for a segment of the hereditary material, within which recombination appears to occur entirely by a mechanism other than crossing over (Whitehouse, 55).

Fig. 2. The polaron hybrid DNA model by Whitehouse (56) showing the mechanism of crossing over. For explanation see text. The site of a mutation is shown by a, and its normal allele by +. Photographically reproduced from H.L.K. Whitehouse, 1965. Crossing over. Sci. Progr. 53, p. 292.


(i)
(ii)
(iii)
(iv)
(v)
(vi)
(vii)
(viii)
a

its complement from the other molecule (Fig. 2(v)). Following correction of heterozygosity and the filling of any gaps with complementary nucleotides (Fig. 2(vi)), and the breakdown, shown by dotted lines, of unpaired chains (Fig. 2(vii)), the crossover is complete (Fig. 2(viii)). There is no net synthesis of DNA, since the breakdown at (vii) equals the synthesis at (iii)" (Whitehouse, 55, P. 291).

It is evident that the above model would fit the origin of the 1:3:4 type of ascus obtained in the crosses with the bisexual strain FGSC 991. Bisexuality, therefore, would be due to DNA heterozygosity of the two chromatids of chromosome I. Correction of this hybrid DNA (located at the mating-type polaron) would yield two chromatids with the following constitution: A/A or a/a. In addition, it would be expected that repair initiated by both strands involved (A as well as a) would result in homozygous, but incomplete A DNA or, in homozygous, but incomplete a DNA. The mating-type reaction of strains carrying this homozygous, but incomplete mating-type DNA, would most likely be of a "neutral" nature.

From the data contained in Table IV and VI it has been concluded (p. 28) that the genotype A does not occur in the expected frequency (50%). On the basis of the "polaron hybrid DNA" theory the deficiency of A genotypes might be explained as being due to preferential repair: viz. homozygous a/a DNA and homozygous "neutral" mating type DNA are the main repair products, whereas homozygous A/A DNA is rarely produced.

Although Whitehouse (55) applies the "polaron hybrid DNA" model as a meiotic recombinational mechanism (assumed to occur during pachytene), the author believes that the theory can be extended to include mitosis. The essential point in the Whitehouse model is the presence of two chromatids. This criterion, however, is not a characteristic of pachytene only. During late mitotic interphase, DNA is duplicated and hence, a situation arises which is comparable to DNA replication at pachytene. Correction of heterozygous DNA prior to mitosis could, therefore, explain the different genotypes (with regard to mating-type) in somatic cells (conidia). However, in order to account for the data obtained (Table III), the above somatic recombinational system requires a feed-back system which gives rise to new hybrid DNA. Continuous repair would otherwise completely deplete the genotype A/a in the vegetative part of the organism. From the data contained in Table III it can be concluded that the genotype A/a is still present in the conidia in an appreciable amount after many mitotic divisions. It is therefore proposed that somatic recombination as outlined by Weijer and Dowding (51) and Mitchell (26) may underly the origin of the A/a genotype in the conidia. Hence, according to this proposal, hybrid DNA would be regenerated in the organism by means of somatic recombination.

Olive (30) draws attention to the possibility that the mating-type factor may consist of a compound locus. Raper (36) and Raper and Esser (37), on the other hand, strictly adhere to a classical "two allele" concept. From the experiments reported in

this thesis, it would seem that the mating-type locus has a compound structure. The origin of the bisexual strain FGSC 991 (p. 11) is believed to be due to mutation of the A genotype by means of UV irradiation of conidia. Since no a genotypes were present in the conidia prior to irradiation, the mutation probably does not involve a duplication of the mating-type locus, or a chromosomal rearrangement, but merely a few mis-paired nucleotides or a short deletion. Hence, impairment of the A allele may have given rise to the a allele. In other words, the A allele probably contains more nucleotides than the a allele. The above view that the mating-type locus may have a compound structure fully agrees with the implications of the "polaron hybrid DNA" theory as advanced by Whitehouse (55).

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